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(54) **Method of automatically selecting Oligonucleotide Hybridization probes**

(57) A method is provided for automatically selecting oligonucleotide hybridization probes for detecting a mutation causing a mismatch in a DNA duplex. This method comprising the steps of: defining the mutation to be detected by a DNA sequence of a mutant DNA strand and a DNA sequence of a wild-type DNA strand corresponding to the mutant DNA strand; defining an integer variable L1 for a first length of the oligonucleotide hybridization probes from the place of mutation in a first direction; defining a range from m1 to n1 of possible values of the variable L1; defining an integer variable L2 for a second length of the oligonucleotide hybridization probes from the place of mutation in a second direction opposite to the first direction; defining a range from m2 to n2 of possible values of the variable L2; selecting one or more kind(s) of the oligonucleotide hybridization probes from probes which have an one-to-one hybridization fit to the mutant DNA strand, probes which have an one-to-one hybridization fit to the wild-type DNA strand corresponding to the mutant DNA strand, probes which have an one-to-one hybridization fit to an opposite sense mutant DNA strand which is the complementary DNA strand to the mutant DNA strand, and probes which

have an one-to-one hybridization fit to an opposite sense wild-type DNA strand which is the complementary DNA strand to the wild-type DNA strand corresponding to the mutant DNA strand; determining all possible hybridization probes from the possible values of the variables L1 and L2, and the kind(s) of probes; defining a thermodynamic nearest-neighbour model for calculating a melting point of a certain hybridization probe hybridized to a DNA strand, the melting point being a temperature at which a predetermined percentage of a multitude of identical pairs of said certain hybridization probe and said DNA strand is in an annealed state; calculating, on basis of the thermodynamic nearest-neighbour model, for all possible hybridization probes a first melting point of the respective probe hybridized with its complementary mutant DNA strand or opposite sense mutant DNA strand, respectively, a second melting point of the respective probe hybridized with its complementary wild-type DNA strand or opposite sense wild-type DNA strand, respectively, and a temperature difference between the second melting point and the first melting point; and ranking the possible hybridization probes with regard to the temperature difference.

EP 1 103 910 A1

## Description

[0001] The invention relates to a method of automatically selecting oligonucleotide hybridization probes for detecting a mutation causing a mismatch in a DNA duplex.

[0002] The hybridization of oligonucleotide hybridization probes is a technique with widespread application in molecular biology. It has been used for the detection of immobilized nucleic acids in northern or southern blotting and for allele specific oligonucleotide hybridization. Provided stringency conditions are met, only a perfect complementary hybridization probe will basepair with the DNA strand observed. The method can therefore be used for mutation detection when the presence of a mismatch caused by a base exchange disrupts the Watson-Crick pairing and destabilizes the partial duplex formed by the hybridization probe and the DNA strand observed.

[0003] A known possible tool for monitoring the hybridization of a labeled hybridization probe to a DNA strand to be observed in an homogenous assay is the LightCycler (Trademark, manufactured by Roche Molecular Biochemicals). Three different wavelengths are available for the optical detection of oligonucleotide hybridization when an appropriate fluorescence resonance energy transfer (FRET) pair is formed by adjacent hybridization of two dye labeled hybridization probes with the DNA strand to be observed. One of the two hybridization probes is called the anchor probe while the other probe which is directly sensitive to the mutation to be detected is called the detection probe. The monitored fluorescence signal is proportional to the amount of the hybridized hybridization probe pairs. The temperature at which a certain percentage (normally 50%) of the hybridization probe pairs is hybridized or in a so-called annealed state is called the melting point  $T_m$  of the hybridization probe pair. This melting point can also be regarded as the melting point of only the detection probe as the melting point of the anchor probe is typically set to be considerably higher than that of the detection probe.

[0004] For mutation detection the  $T_m$  shift caused by the mutation should be as high as possible to ensure good discrimination of heterozygotes.

[0005] General guidelines for the construction of hybridization probes for use in both, quantification and mutation detection, have been given [1], but these depend on an accurate estimation of DNA melting temperatures.

[0006] The melting temperature of short (<20 bp) oligonucleotides is often estimated with the Wallace/Ikatura rule  $T_m = 2^\circ\text{C} (\text{A-T bp}) + 4^\circ\text{C} (\text{G-C bp})$ . Although this approximation assumes a salt concentration of 0.9M NaCl, typical for dot blots and other hybridizations, it also works well for PCR applications which are not very sensitive to different  $T_m$ s. However, for hybridization probes a more accurate estimation of  $T_m$  is required.

[0007] From US 3 556 749 it is known to automatically select oligonucleotide hybridization probes taking their respective melting points into consideration. However, this known method proves not to be accurate.

[0008] Therefore, it is an object of this invention to provide a method of automatically selecting oligonucleotide hybridization probes for detecting a mutation causing a mismatch in a DNA duplex by a sufficient accurate estimation of their  $T_m$ .

[0009] This object is achieved by a method according to claim 1.

[0010] Advantageous embodiments of this method are outlined in the subclaims 2 to 9.

[0011] The nearest-neighbor (n-n) model is based on thermodynamic calculations and gives the most precise prediction of oligonucleotide stability. This model assumes that the thermodynamic parameters for a given pair depend only on the identity of its adjacent pair and that these nearest neighbor parameters are pairwise additive. The stability of a given oligonucleotide sequence is a function of two basic sources, the sum of the interstrand H bonding between Watson-Crick paired bases and the intrastrand base stacking. The application of this model for calculation of DNA duplex stability will be detailed in the methods section of this chapter. The derivation of the used formulas can be found in the literature [2] and will not be given here. Several data sets are now available that describe the ten n-n pairs that occur in double stranded oligonucleotide DNA [3-6]. The contribution of a mismatch to the duplex stability depends on the location and orientation and on the neighboring bases as well [3]. The destabilizing effect of the 48 possible single mismatches can also be taken into account if the n-n data of the respective mismatches are used for the calculation of the melting temperature [3;7-10]. Oligonucleotides with repetitive sequences or strings of A-T base pairs may deviate from the n-n model as well as molecules which do not melt in a two state (all or nothing) manner [11]. Hybridization conditions (ionic strength and probe concentration) also influence the  $T_m$  and must be considered. It could be proved that the n-n model is able to predict the melting points observed with hybridization probe assays with a standard error of less than  $1^\circ\text{C}$ . The n-n model is most beneficial for the thermodynamic predictions of oligonucleotide DNA. In longer DNA strands, interactions that are independent of the neighboring bases become increasingly important. This reduces the utility of the n-n model for the prediction of longer (>150 bp) DNA duplexes.

[0012] In the following the invention will be described in more detail with regard to preferred embodiments of the new method. Further, a brief description on how thermodynamic data for use in the new method can be calculated will be given. Next, the application of the n-n model to hybridization probe assays on the LightCycler will be validated. At last a general comment will be given on sensitivity and specificity of hybridization probes selected by the new method.

[0013] In the Figures:

Figure 1 is a flux diagram of a first embodiment of the new method;  
 Figure 2 is a flux diagram of a second embodiment of the new method;  
 Figure 3 is a flux diagram of a third embodiment of the new method;  
 Figure 4 shows an example DNA duplex of a 18mer oligonucleotide; there are 17 n-n doublets and initiation en-  
 5 ergies for termination with G-C and A-T must be considered; the oligonucleotide is non-self-complementary;  
 Figure 5 is a diagram of experimentally observed melting points versus melting points calculated with the n-n  
 model; the regression was calculated according to Passing and Bablok [33], while the standard error of the estimate  
 ( $S_{y|x}$ ) was calculated with the principal component method; and  
 Figure 6 is a contour plot showing the interdependence of the melting temperature shift of 19mer probes destabi-  
 10 lized by a single mutation versus their GC content; the different frequencies are shown in shadings.

[0014] As indicated in Figure 1, a method of automatically selecting oligonucleotide hybridization probes for detecting  
 a mutation causing a mismatch in a DNA duplex starts with the step 1 of defining the mutation to be detected by a DNA  
 sequence of a mutant DNA strand and a DNA sequence of a wild-type DNA strand corresponding to the mutant DNA  
 15 strand. Here, it is unimportant whether the mutant DNA strand and the wild-type DNA strand are sense or antisense  
 strands as the sense strand always defines the antisense strand and vice versa. In the following as well as in the claims  
 a term opposite sense strand is used to refer to the strand having the sense opposite to the strand defined in step 1.  
 Therefore, opposite sense strand is no other formulation for antisense strand but may also refer to a sense strand.

[0015] In step 2 of the method integer variables L1 and L2 are defined for a first and a second lengths of the oligo-  
 nucleotide hybridization probes from the place of mutation in a first and a second direction opposite to the first direction.  
 20 Further, ranges from m1 to n1 and from m2 to n2 of possible values of the variables L1 and L2, respectively, are defined.

[0016] Alternatively, step 2 can include defining a first variable for an end point of the oligonucleotide hybridization  
 probes being located from the place of mutation in a first direction; and defining a second variable for a length of the  
 hybridization probes in a second direction opposite to the first direction. Then, first and second ranges are to be  
 25 defined for the first and the second variables, in which the second range is dependent of the first range as the hybrid-  
 ization probes have in any case to cover the place of mutation.

[0017] At least in the end, both alternatives of step 2 have the same results. Therefore, they are in the following as  
 well as in the claims regarded as identical.

[0018] In step 3 one or more kind(s) of the oligonucleotide hybridization probes are selected from

30 probes which have an one-to-one hybridization fit to the mutant DNA strand,  
 probes which have an one-to-one hybridization fit to the wild-type DNA strand corresponding to the mutant DNA  
 strand,  
 probes which have an one-to-one hybridization fit to an opposite sense mutant DNA strand which is the comple-  
 35 mentary DNA strand to the mutant DNA strand, and  
 probes which have an one-to-one hybridization fit to an opposite sense wild-type DNA strand which is the com-  
 plementary DNA strand to the wild-type DNA strand corresponding to the mutant DNA strand.

All of the above kinds can be selected, if no special requirements are given. If such requirements exist, special kinds  
 40 of the oligonucleotide hybridization probes can be selectively selected or deselected.

[0019] In step 4 all possible hybridization probes are determined from the input data of steps 2 and 3, i.e. from the  
 possible values of the variables L1 and L2, and the kind(s) of probes.

[0020] In step 5 a thermodynamic nearest-neighbor (n-n) model for calculating a melting point of a certain hybrid-  
 45 zation probe hybridized to a DNA strand is defined. That n-n model will be discussed in detail below. The melting point  
 is a temperature at which a predetermined percentage of a multitude of identical pairs of said certain hybridization  
 probe and said DNA strand is in an annealed state, i.e. hybridized.

[0021] In step 6, on basis of the thermodynamic nearest-neighbor model, the following values are calculated for all  
 possible hybridization probes:

50 a first melting point of the respective probe hybridized with its complementary mutant DNA strand or opposite  
 sense mutant DNA strand, respectively,  
 a second melting point of the respective probe hybridized with its complementary wild-type DNA strand or opposite  
 sense wild-type DNA strand, respectively, and  
 a temperature difference between the second melting point and the first melting point.  
 55

[0022] At last, in step 7, the possible hybridization probes are ranked with regard to the temperature difference, and  
 a ranked list of the hybridization probes is output.

[0023] According to Figure 2, a preferred embodiment of the new method further includes step 8 in which a temper-

ature range of a hybridization assay to be employed for detecting the mutation using the oligonucleotide hybridization probes is defined. Then in step 9 following step 6 and preceding step 7, it is checked for each of the possible hybridization probes whether both the first and the second melting points are within the temperature range of the hybridization assay. Here, in step 7 only those possible hybridization probes are ranked which have both their melting points within the temperature range of the hybridization assay.

[0024] As the maximum  $T_m$  shift caused by the mutation is limited, it is also sufficient to define a narrower temperature range in step 8, and to only check whether that melting point corresponding to the narrower temperature range is within said temperature range.

[0025] The actual temperature range is dependent of the hybridization assay to be employed. However, it will normally be defined within a range from 20 to 80 °C.

[0026] According to Figure 2, a further step 10 is added to the method according to Figure 1. In step 10 at least one physico-chemical parameter of the hybridization assay to be employed for detecting the mutation using the oligonucleotide hybridization probes is additionally defined. Correspondingly, step 5 includes defining the thermodynamic nearest-neighbor model dependently of the at least one physico-chemical parameter of the hybridization assay. The at least one physico-chemical parameter of the hybridization assay can for example be an ion concentration under which the hybridization of the hybridization probes is to be monitored. Such an ion concentration can be a total cation concentration denoted as a  $Na^+$  cation equivalent concentration.

[0027] According to Figure 3, a further step 11 is added to the method according to Figure 2. In step 10 an integer number  $N$  of the hybridization probes to be automatically selected is defined. Correspondingly, in step 7 only a list of the  $N$  top ranked possible hybridization probes is output. Alternatively, in step 7 the possible hybridization probes can be separately ranked for each of the kinds of the oligonucleotide hybridization probes, and the  $N$  top ranked possible hybridization probes can be output for each of the kinds of the oligonucleotide hybridization probes.

[0028] Further, step 7 can include checking, for each of the possible hybridization probes, whether it is able to hybridize either to the mutant DNA strand and the opposite sense mutant DNA strand, or to the wild-type DNA strand and the opposite sense wild-type DNA strand in more than one way. Then, ranking the possible hybridization probes with regard to the temperature difference may include ranking only those possible hybridization probes which are neither able to hybridize to the mutant DNA strand and the opposite sense mutant DNA strand, nor to the wild-type DNA strand and the opposite sense wild-type DNA strand in more than one way.

[0029] In an optional step 12 one or more further mutations which may occur on the mutant DNA strand or the wild-type DNA-strand or the opposite sense wild-type DNA strand may be defined, so that in step 7 it may be checked, for each of the possible hybridization probes, whether it is sensitive to the further mutation in the same way as to the mutation. Thus ranking the possible hybridization probes with regard to the temperature difference may be limited to ranking only those possible hybridization probes which are not sensitive to the further mutation in the same way as to the mutation.

[0030] Further refinements due to special requirements for the possible hybridization probes to be selected are possible and easily accomplished by those skilled in the art.

[0031] Now, a theoretical background is given for the  $n$ -n Model to be defined in step 5 of the methods according to Figures 1 to 3.

[0032] The enthalpy ( $\Delta H^\circ$ ) and entropy ( $\Delta S^\circ$ ) of an oligonucleotide duplex is needed for the calculation of its melting temperature:

$$T_m = \frac{\Delta H^\circ}{\Delta S^\circ [Na^+] + R \times \ln(CT)} - 273.15 \quad [\text{Eq 1}]$$

where  $R$  is the gas constant (1.987 cal/K $\times$ mol),  $CT$  is the strand concentration,  $\Delta S^\circ [Na]$  is the entropy at a given sodium equivalent concentration. For non-self-complementary sequences  $CT$  is the concentration of oligonucleotides divided by 4. A sequence is considered to be self-complementary if two strands of the same sequence can form a duplex.

[0033] For the calculation of  $T_m$   $\Delta H^\circ$  needs to be calculated from Eq 2 and  $\Delta S^\circ [Na]$  from Eqs 3-5 knowing  $CT$ . According to the  $n$ -n model, energy for helix formation is the sum of three terms, one for helix propagation energy from the sum of every base pair, one for an energy change caused by helix initiation and one to account for effects caused by duplex formation of self-complementary strands. The following formula applies for the calculation of  $\Delta H^\circ$  for an oligonucleotide:

$$\Delta H^\circ_{\text{total}} = \sum_i n_i \Delta H^\circ(i) + \Delta H^\circ(5'\text{init}) + \Delta H^\circ(3'\text{init}) + \Delta H^\circ(\text{symmetry}) \quad [\text{Eq 2}]$$

$\Delta H^0(i)$  is the enthalpy change for the ten possible n-n and  $n_i$  is the number of occurrences of the respective doublet.

[0034] For  $\Delta S^0$  a similar formula applies:

$$\Delta S^0_{\text{total}} = \sum_i n_i \Delta S^0(i) + \Delta S^0(5'\text{init}) + \Delta S^0(3'\text{init}) + \Delta S^0(\text{symmetry}) \quad [\text{Eq 3}]$$

[0035] For Eq 2 and Eq 3 it should be noted, that the symmetry correction is zero for non-self-complementary oligonucleotides. The  $\Delta H^0$  and  $\Delta S^0$  values for Watson-Crick nearest neighbors with matched and mismatched basepairs as well as those for initiation and symmetry correction were taken from published data and are summarized in tables 1 and 2. A standard PCR buffer contains 50 mM KCl and 1.5 mM  $\text{Mg}^{2+}$ , for rapid cycling applications higher Mg concentrations are often used.  $\text{Mg}^{2+}$  cations have a 140 times higher stabilizing effect on duplexes compared to  $\text{Na}^+$  [13]. The concentrations of the monovalent ions do not add much to this effect, at least under the conditions which apply for PCR [13]. Therefore the sodium equivalents are calculated as follows:

$$\text{Na}^+ \text{ equivalent} = [\text{Mg}^{2+}] \times 140 \quad [\text{Eq 4a}]$$

[0036] Alternatively a formula based on a 100 times higher DNA stabilizing effect of  $\text{Mg}^{2+}$  cations may be used. This also takes into account the monovalent ions [14]:

$$\text{Na}^+ \text{ equivalent} = [\text{Mg}^{2+}] \times 100 + \text{monovalent ions} \quad [\text{Eq 4b}]$$

[0037] The entropy is salt dependent and therefore  $\Delta S^0$  must be corrected if the sodium concentration is different from 1M NaCl, the condition under which the parameters tabulated in table 1 were derived [5].

$$\Delta S^0[\text{Na}] = \Delta S^0[1\text{M Na}] + 0.368 \times N \times \ln[\text{Na}^+] \quad [\text{Eq 5}]$$

where N is equal to the oligonucleotide length minus 1.

If DMSO is included in a PCR reaction, it induces a  $T_m$  reduction of 0.6°C for each percent DMSO [15].

#### 1. Example: Calculation of $T_m$ for a 18mer without mismatches

[0038] As an example the  $T_m$  of the 18 mer

5'-ACG ATG GCA GTA GCA TGC-3' according to Figure 4 is calculated.  
3'-TGC TAC CGT CAT CGT ACG-5'

[0039] This example oligonucleotide has 17 n-n doublets. These are AC/TG, CG/GC, GA/CT, AT/TA, TG/AC, GG/CC, GC/CG and so on, see Figure 4.

It should be noted that the first doublet (AC/TG) is identical with GT/CA. Only the thermodynamic data for latter doublet is listed in table 1 to save space. Palindromic sequences are not listed twice. From the n-n given in table 1, the number of different doublets found in the above 18mer oligonucleotide are as follows:

$N_{AA/TT} 0$ ,  $N_{AT/TA} 2$ ,  $N_{TA/AT} 1$ ,  $N_{CA/GT} 4$ ,  $N_{GT/CA} 2$ ,  $N_{CT/GA} 2$ ,  $N_{GA/CT} 1$ ,  $N_{CG/GC} 1$ ,  $N_{GC/CG} 3$ ,  $N_{GG/CC} 1$

[0040] With data from table 1  $\Delta H^0$  can be calculated according to Eq 2:

$$\sum_i n_i \Delta H^0(i) \text{ results in } 0 \times (-7.9) + 2 \times (-7.2) + 1 \times (-7.2) + 4 \times (-8.5) + 2 \times (-8.4) + 2 \times (-7.8) + 1 \times (-8.2) + 1 \times (-10.6) + 3 \times (-9.8) + 1 \times (-8.0) = -144.2 \text{ kcal/mol}$$

[0041] The initiation term for each direction is 0.1 kcal/mol for initiation with terminal G-C and 2.3 kcal/mol for initiation with terminal A-T.

No symmetry correction is necessary since the oligonucleotide is non-self-complementary.  $\Delta H^0_{\text{total}} = -144.2 + 0.1 + 2.3 \text{ kcal/mol} = -141.8 \text{ kcal/mol}$ .

[0042]  $\Delta S^0$  calculation according to Eq 3:

$$\sum_i n_i \Delta S^0(i) \text{ results in } 0 \times (-22.2) + 2 \times (-20.4) + 1 \times (-21.3) + 4 \times (-22.7) + 2 \times (-22.4) + 2 \times (-21.0) + 1 \times (-22.2) + 1 \times (-27.2) + 3 \times (-24.4) + 1 \times (-19.9) = -382.2 \text{ cal/Kxmol}$$

The initiation term for each direction is -2.8 cal/Kxmol for initiation with terminal G-C and 4.1 cal/Kxmol for initiation with terminal A-T, total +1.3 cal/Kxmol.

No symmetry correction is necessary since the oligonucleotide is non-self-complementary.

$$\Delta S_{\text{total}}^0 = -380.9 \text{ cal/Kxmol}$$

[0043] Sodium equivalents calculation according to Eq 4a:

Assuming the PCR contains 2.5 mM  $\text{Mg}^{2+}$ , according to Eq 4a the sodium equivalent is  $2.5 \text{ mM} \times 140 = 350 \text{ mM} = 0.35 \text{ M}$

[0044] Calculating  $\Delta S$  for a given sodium equivalent according to Eq 5:

For a 18 bp duplex is  $N=17$ .

$$\Delta S^* [\text{Na}] = -380.9 \text{ cal/Kxmol} + 0.368 \times 17 \times \ln [0.35] = -387.5 \text{ cal/Kxmol}$$

[0045] Calculating  $T_m$  according to Eq 1

Because of the different units  $\Delta H$  must be multiplied by 1000 to cal/mol. To convert from Kelvin to Celsius subtract 273.15.

The concentration of the probe is  $0.1 \mu\text{M} = 0.1 \times 10^{-6} \text{ M}$  and this must be divided by 4 because the sequence is non-self-complementary.

$$T_m = \frac{-141800 \text{ cal/mol}}{-387.5 \text{ cal/K} \times \text{mol} + 1.987 \text{ cal/K} \times \text{mol} \times \ln\left(\frac{0.1 \times 10^{-6} \text{ mol}}{4}\right)} - 273.15 = 62.7^\circ \text{C}$$

## 2. Example: Calculation of $T_m$ for a 18mer with mismatches

[0046] As an example the same 18 mer 5'-ACG ATG GCA GTA GCA TGC-3' as above is taken assuming a G->A mutation at the third base. Hybridization of the wild type probe with the mutated strand leads to the following duplex with a G-T mismatch in the trimer CGA/GTT:

5'-ACG ATG GCA GTA GCA TGC-3'

3'-TGI TAC CGT CAT CGT ACG-5'

[0047] The oligonucleotide example has 15 Watson-Crick paired doublets (table 1) and 2 mismatched doublets (table 2). These are AC/TG, CG/GT, GA/TT, AT/TA, TG/AC, GG/CC, GC/CG and so on. The number of different doublets are:  $N_{AA/TT} 0$ ,  $N_{AT/TA} 2$ ,  $N_{TA/AT} 1$ ,  $N_{CA/GT} 4$ ,  $N_{GT/CA} 2$ ,  $N_{CT/GA} 1$ ,  $N_{GA/CT} 1$ ,  $N_{CG/GC} 0$ ,  $N_{GC/CG} 3$ ,  $N_{GG/CC} 1$ . The two doublets affected by the mutation are:  $N_{CG/GT}$  and  $N_{GA/TT}$ . Each of these doublets occurs once. The remaining n-n pairs are equal to example 1.

[0048] With data also from table 2  $\Delta H^0$  can be calculated according to Eq 2:

For the Watson-Crick paired bases  $\sum_i n_i \Delta H^0(i)$  results in  $0 \times (-7.9) + 2 \times (-7.2) + 1 \times (-7.2) + 4 \times (-8.5) + 2 \times (-8.4) + 2 \times (-7.8) + 0 \times (-8.2) + 0 \times (-10.6) + 3 \times (-9.8) + 1 \times (-8.0) = -125.4 \text{ kcal/mol}$ . Two doublets are affected by mismatches and their thermodynamic properties are found in table 2. For the CG/GT doublet  $\Delta H^0 = -4.1 \text{ kcal/mol}$ , for GA/TT (look up TT/GA in table 2, this is the same as GA/TT)  $\Delta H^0 = -1.3 \text{ kcal/mol}$ .

Furthermore, the initiation term is added for each direction, that is 0.1 kcal/mol for initiation with terminal G-C and 2.3 kcal/mol for initiation with terminal A-T.

No symmetry correction is necessary as the oligonucleotide is non-self-complementary.

$$\Delta H^{\circ}_{\text{total}} = -125.4 - 4.1 - 1.3 + 0.1 + 2.3 = -128.4 \text{ kcal/mol}$$

[0049]  $\Delta S^{\circ}$  calculation according to Eq 3:

For the Watson-Crick paired bases  $\sum_i n_i \Delta S^{\circ}(i)$  results in  $0 \times (-22.2) + 2 \times (-20.4) + 1 \times (-21.3) + 4 \times (-22.7) + 2 \times (-22.4) + 2 \times (-21.0) + 0 \times (-22.2) + 0 \times (-27.2) + 3 \times (-24.4) + 1 \times (-19.9) = -332.8 \text{ cal/K} \times \text{mol}$ . Two doublets are affected by mismatches and their thermodynamic properties are again found in table 2. For the CG/GT doublet  $\Delta S^{\circ} = -11.7 \text{ kcal/K} \times \text{mol}$ , for GA/TT (look up TT/GA in table 2, this is the same as GA/TT)  $\Delta S^{\circ} = -5.3 \text{ kcal/K} \times \text{mol}$ .

The initiation term is added for each direction, that is  $-2.8 \text{ cal/K} \times \text{mol}$  for initiation with terminal G-C and  $4.1 \text{ cal/K} \times \text{mol}$  for initiation with terminal A-T.

No symmetry correction is necessary as the oligonucleotide is non-self-complementary.  $\Delta S^{\circ}_{\text{total}} = -348.5 \text{ cal/K} \times \text{mol}$

[0050] Sodium equivalents calculation according to Eq 4a:

[0051] Conditions are the same as above:  $2.5 \text{ mM Mg}^{2+}$ , according to Eq 4a the sodium equivalent is  $2.5 \text{ mM} \times 140 = 350 \text{ mM} = 0.35 \text{ M}$ .

[0052] Calculating  $\Delta S^{\circ}$  for a given sodium equivalent according to Eq 5:

For a 18 bp duplex is  $N=17$ .

$$\Delta S^{\circ} [\text{Na}] = -348.5 \text{ cal/K} \times \text{mol} + 0.368 \times 17 \times \ln [0.35] = -355.1 \text{ cal/K} \times \text{mol}$$

[0053] Calculating  $T_m$  according to Eq 1

Again  $\Delta H^{\circ}$  must be multiplied with 1000 to change to cal/mol. 273.15 is subtracted to convert from Kelvin to Celsius.

The concentration of the probe is  $0.1 \mu\text{M} = 0.1 \times 10^{-6} \text{ M}$  and this must be divided by 4 because the sequence is non-self-complementary.

$$T_m = \frac{-128400 \text{ cal/mol}}{-355.1 \text{ cal/K} \times \text{mol} + 1.987 \text{ cal/K} \times \text{mol} \times \ln \left( \frac{0.1 \times 10^{-6} \text{ mol}}{4} \right)} - 273.15 = 56.2^{\circ}\text{C}$$

### 3. Example: Calculation of $\Delta T_m$ for a hybridization probe mutation detection assay

[0054] In the first example a  $T_m$  of  $62.7^{\circ}\text{C}$  was calculated for a 18mer probe. In the second calculation a lower  $T_m$  of  $56.2^{\circ}\text{C}$  was calculated due to a single mismatch caused by a G->A mutation. If the former probe were to hybridize on the latter mutated strand, a calculated reduction in  $T_m$  of  $6.5^{\circ}\text{C}$  would result.

[0055] The thermodynamic calculations assume that the annealing occurs at pH 7.0. This is not the case in standard PCR buffer systems, but the resulting error seems to be small. More important is to note that mismatches in the terminal and penultimate position are not properly reflected by the parameters in table 2 [3;10]. The thermodynamic data set for double mismatches (i.e., immediately adjacent mismatches) is still incomplete. Only for the few double mismatches listed in table 2 is a reliable prediction possible. The influence of the salt concentration on oligonucleotide stability is also still under investigation [4;5;13]. Nevertheless, the methods presented here will allow a reliable estimate of hybridization probe  $T_m$  on the LightCycler but may require correction of the sodium equivalents according to the local PCR conditions.

### 4. Validation of the n-n model on the LightCycler

[0056] The formulas described above were incorporated into a Microsoft® Excel® spreadsheet software which is capable of calculating the thermodynamic data for matched and mismatched oligonucleotides under different concentrations and ionic strengths. This program is available for download via <http://server1.medikc.med.uni-goettingen.de/meltcalc.htm> or it may be requested by email. A total of 87 melting points resulting from eleven different published [16-26] and 14 unpublished hybridization probe assays were calculated and showed an excellent fit with the predictions from the n-n model, as may be seen from Figure 5. For the data shown in Figure 5 Eq. 4b was used to calculate the  $\text{Na}^+$  equivalents which gave a better fit when using the published  $T_m$  data from different laboratories. However, under the standard PCR conditions used with  $2.5 \text{ mM Mg}^{2+}$  and 5% DMSO the best prediction were obtained using

Eq 4a.

[0057] Genotyping assays for the apolipoprotein B-3500 and factor V Leiden mutation have been performed. For both loci different mutations have been described that occur under the detection probe. These are the G10699A and C10698T mutations of apolipoprotein B [27] and the G1691A, A1692C and G1689A mutations for factor V [20;28].

5 Increasing knowledge on the prevalence of single nucleotide polymorphisms in the human genome [29;30] raises the important question about the sensitivity of hybridization probe assays towards different mutations under a probe. Melting point calculations for different possible mutations under a 19mer probe led us speculate that no mutation under a properly designed probe will remain undetected. However, there are different mutations possible that destabilize the probe binding to the same extent [12]. These mutations will not be discriminated from each other. Fig. 3 shows how 10 19mer probes with different GC content are destabilized by a single base mutation. The most important trend is that probes with higher GC content show less  $T_m$  shift due to a single mutation. Another principal point is that shorter probes show higher  $T_m$  shifts because the destabilizing impact of the mutation on the total stability is higher in shorter duplexes. The thermodynamic n-n model provides the theoretical basis for the stability of matched and mismatched oligonucleotides. The comparison of experimentally derived melting points with those calculated from the n-n model 15 show very good agreement.

Using a detection probe that has been properly optimized according to this model it may be appreciated that the detection of every point mutation should be possible. However, it is possible that different mutations under a probe result in the same  $T_m$  reduction. Important factors for the design of hybridization probes are: (I.) Absence of significant self- or cross dimerization of probes and amplification primers. (II.) Equal melting points of detection and anchor probe and 20 absence of false priming sites on the amplicon for hybridization probes used for quantification. (III.) Use of a detection probe with a maximum  $T_m$  shift caused by the investigated mutation (see below) and an anchor probe with a  $T_m$  approximately 10% higher than that of the detection probe for mutation detection. Provided it is not in the penultimate or ultimate position the place where the mutation is located under the probe is not important. The resulting melting point shift will be affected by the type of mutation and the neighboring bases but not significantly by the position itself. 25 The commonest mutations are G→A mutations [31]. However, the resulting G:T mismatch under the probe is among the most stable mismatches which occur together with G:A and G:G mismatches [9]. It is therefore advisable to place the detection probe on the antisense strand. The resulting complementary C:A mismatch has a higher destabilizing effect and is more readily detected. (IV.) The gap between the fluorophores should be as small as possible since the FRET efficiency is inversely proportional to the sixth power of the distance between the donor and the acceptor dye 30 [32]. One to five basepairs gap has been reported to give good results [1].

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Table 1.

Unified parameters for oligonucleotide  $\Delta H^\circ$  and  $\Delta S^\circ$  in 1 M NaCl [5].  
(Nearest neighbor dimer duplexes are presented with a slash separating strands in antiparallel orientation, e.g. AT/TA means 5'-AT-3' Watson-Crick paired with 3'-TA-5'. )

Sequence	$\Delta H^\circ$ kcal/mol	$\Delta S^\circ$ cal/Kxmol
AA/TT	-7.9	-22.2
AT/TA	-7.2	-20.4
TA/AT	-7.2	-21.3
CA/GT	-8.5	-22.7
GT/CA	-8.4	-22.4
CT/GA	-7.8	-21.0
GA/CT	-8.2	-22.2
CG/GC	-10.6	-27.2
GC/CG	-9.8	-24.4
GG/CC	-8.0	-19.9
Initiation with terminal G-C	0.1	-2.8
Initiation with terminal A-T	2.3	4.1
Symmetry correction for self complimentary oligos	0	-1.4

Table 2.

Parameters for mismatched oligonucleotide  $\Delta H^\circ$  and  $\Delta S^\circ$  in 1 M NaCl.  
(References are given in the table. Nearest neighbor dimer duplexes are presented with a slash separating strands in antiparallel orientation, e.g. AT/TG means 5'-AT-3' mismatched with 3'-TG-5'. Mismatches are in the second position of the listed doublet except for double mismatches. These parameters are not valid for the calculation of mismatches in the terminal or penultimate position.)

Sequence	$\Delta H^\circ$ kcal/mol	$\Delta S^\circ$ cal/Kxmol
Internal G-A mismatches [9]		
AA/TG	-0.6	-2.3
AG/TA	-0.7	-2.3
CA/GG	-0.7	-2.3
CG/GA	-4.0	-13.2
GA/CG	-0.6	-1.0
GG/CA	0.5	3.2
TA/AG	0.7	0.7
TG/AA	3.0	7.4
Internal C-T mismatches [8]		
AC/TT	0.7	0.2
AT/TC	-1.2	-6.2
CC/GT	-0.8	-4.5
CT/GC	-1.5	-6.1

EP 1 103 910 A1

Table 2. (continued)

	Sequence	$\Delta H^\circ$ kcal/mol	$\Delta S^\circ$ cal/Kxmol
5	Internal C-T mismatches [8]		
	GC/CT	2.3	5.4
	GT/CC	5.2	13.5
10	TC/AT	1.2	0.7
	TT/AC	1.0	0.7
	Internal A-C mismatches [7]		
	AA/TC	2.3	4.6
15	AC/TA	5.3	14.6
	CA/GC	1.9	3.7
	CC/GA	0.6	-0.6
20	GA/CC	5.2	14.2
	GC/CA	-0.7	-3.8
	TA/AC	3.4	8.0
	TC/AA	7.6	20.2
25	Internal G-T mismatches [3]		
	AG/TT	1.0	0.9
	AT/TG	-2.5	-8.3
30	CG/GT	-4.1	-11.7
	CT/GG	-2.8	-8.0
	GG/CT	3.3	10.4
	GT/CG	-4.4	-12.3
35	TG/AT	-0.1	-1.7
	TT/AG	-1.3	-5.3
	Internal A-A mismatches [10]		
40	AA/TA	1.2	1.7
	CA/GA	-0.9	-4.2
	GA/CA	-2.9	-9.8
	TA/AA	4.7	12.9
45	Internal C-C mismatches [10]		
	AC/TC	0.0	-4.4
	CC/GC	-1.5	-7.2
50	GC/CC	3.6	8.9
	TC/AC	6.1	16.4
	Internal G-G mismatches [10]		
55	AG/TG	-3.1	-9.5
	CG/GG	-4.9	-15.3
	GG/CG	-6.0	-15.8

Table 2. (continued)

Sequence	$\Delta H^\circ$ kcal/mol	$\Delta S^\circ$ cal/Kxmol
Internal G-G mismatches [10]		
TG/AG	1.6	3.6
Internal T-T mismatches [10]		
AT/TT	-2.7	-10.8
CT/GT	-5.0	-15.8
GT/CT	-2.2	-8.4
TT/AT	0.2	-1.5
Double mismatches [3]		
GG/TT	5.8	16.3
GT/TG	4.1	9.5
TG/GT	-1.4	-6.2

## Claims

1. A method of automatically selecting oligonucleotide hybridization probes for detecting a mutation causing a mismatch in a DNA duplex, the method comprising the steps of:

- defining the mutation to be detected by

- a DNA sequence of a mutant DNA strand and

- a DNA sequence of a wild-type DNA strand corresponding to the mutant DNA strand;

- defining an integer variable L1 for a first length of the oligonucleotide hybridization probes from the place of mutation in a first direction;

- defining a range from m1 to n1 of possible values of the variable L1 ;

- defining an integer variable L2 for a second length of the oligonucleotide hybridization probes from the place of mutation in a second direction opposite to the first direction;

- defining a range from m2 to n2 of possible values of the variable L2;

- selecting one or more kind(s) of the oligonucleotide hybridization probes from

- probes which have an one-to-one hybridization fit to the mutant DNA strand,

- probes which have an one-to-one hybridization fit to the wild-type DNA strand corresponding to the mutant DNA strand,

- probes which have an one-to-one hybridization fit to an opposite sense mutant DNA strand which is the complementary DNA strand to the mutant DNA strand, and

- probes which have an one-to-one hybridization fit to an opposite sense wild-type DNA strand which is the complementary DNA strand to the wild-type DNA strand corresponding to the mutant DNA strand;

- determining all possible hybridization probes from the possible values of the variables L1 and L2, and the kind(s) of probes;

- defining a thermodynamic nearest-neighbor model for calculating a melting point of a certain hybridization probe hybridized to a DNA strand, the melting point being a temperature at which a predetermined percentage of a multitude of identical pairs of said certain hybridization probe and said DNA strand is in an annealed state;

- calculating, on basis of the thermodynamic nearest-neighbor model, for all possible hybridization probes

- a first melting point of the respective probe hybridized with its complementary mutant DNA strand or opposite sense mutant DNA strand, respectively,

- a second melting point of the respective probe hybridized with its complementary wild-type DNA strand

or opposite sense wild-type DNA strand, respectively, and  
a temperature difference between the second melting point and the first melting point; and

- ranking the possible hybridization probes with regard to the temperature difference.

2. The method of claim 1, and further comprising the steps of:

- defining a temperature range of a hybridization assay to be employed for detecting the mutation using the oligonucleotide hybridization probes; and
- checking for each of the possible hybridization probes whether both the first and the second melting points are within the temperature range of the hybridization assay; wherein the step of ranking the possible hybridization probes with regard to the temperature difference includes:
- ranking only those possible hybridization probes which have both their melting points within the temperature range of the hybridization assay.

3. The method of claim 2, wherein the step of defining the temperature range of the hybridization assay to be employed for detecting the mutation includes:

- defining the temperature range within a range from 20 to 80 °C.

4. The method according to any of the claims 1 to 3, and further comprising the step of:

- defining at least one physico-chemical parameter of a hybridization assay to be employed for detecting the mutation using the oligonucleotide hybridization probes; wherein the step of defining a thermodynamic nearest-neighbor model includes:
- defining the thermodynamic nearest-neighbor model dependently of the at least one physico-chemical parameter of the hybridization assay.

5. The method of claim 4, wherein the step of defining at least one physico-chemical parameter of the hybridization assay to be employed for detecting the mutation includes:

- defining at least one ion concentration as one of the physico-chemical parameters.

6. The method of claim 5, wherein the step of defining at least one ion concentration as one of the physico-chemical parameters includes:

- defining a Na<sup>+</sup> cation equivalent concentration.

7. The method according to any of the claims 1 to 6, and further comprising the steps of:

- defining an integer number N of the hybridization probes to be automatically selected; and
- making a list of the N top ranked possible hybridization probes.

8. The method according to one of the claims 1 to 7, wherein the step of ranking the possible hybridization probes with regard to the temperature difference includes:

- ranking the possible hybridization probes separately for each of the kinds of the oligonucleotide hybridization probes.

9. The method according to one of the claims 1 to 8, and further comprising the step of:

- checking, for each of the possible hybridization probes, whether it is able to hybridize either to the mutant DNA strand and the opposite sense mutant DNA strand, or to the wild-type DNA strand and the opposite sense wild-type DNA strand in more than one way;

wherein the step of ranking the possible hybridization probes with regard to the temperature difference includes:

- ranking only those possible hybridization probes which are neither able to hybridize to the mutant DNA strand

and the opposite sense mutant DNA strand, nor to the wild-type DNA strand and the opposite sense wild-type DNA strand in more than one way.

10. The method according to one of the claims 1 to 9, and further comprising the steps of:

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- defining at least one further mutation which may occur on the mutant DNA strand or the wild-type DNA-strand or the opposite sense wild-type DNA strand;
- checking, for each of the possible hybridization probes, whether it is sensitive to the further mutation in the same way as to the mutation;

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wherein the step of ranking the possible hybridization probes with regard to the temperature difference includes:

- ranking only those possible hybridization probes which are not sensitive to the further mutation in the same way as to the mutation.

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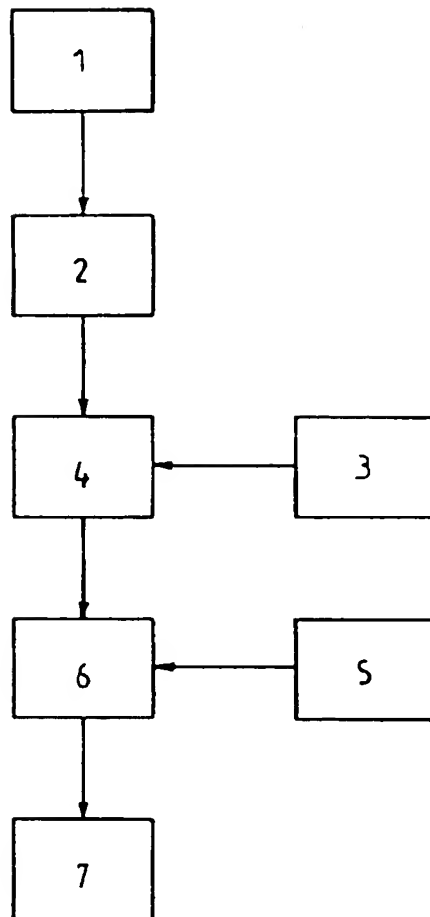


Fig. 1

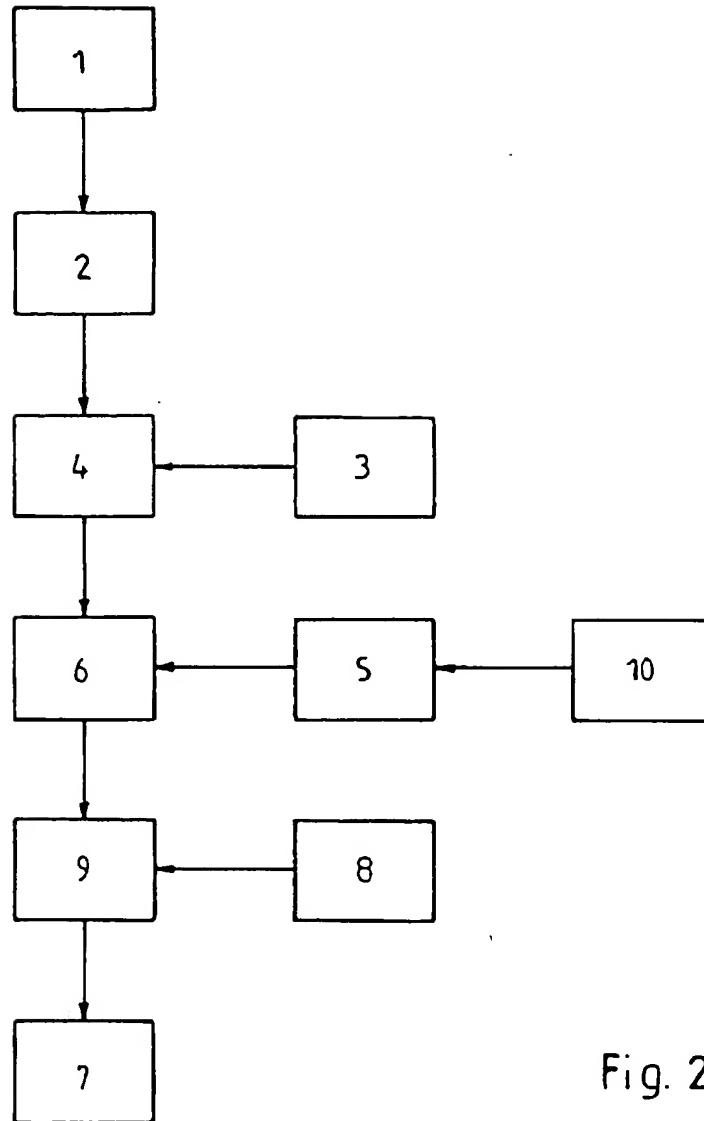


Fig. 2



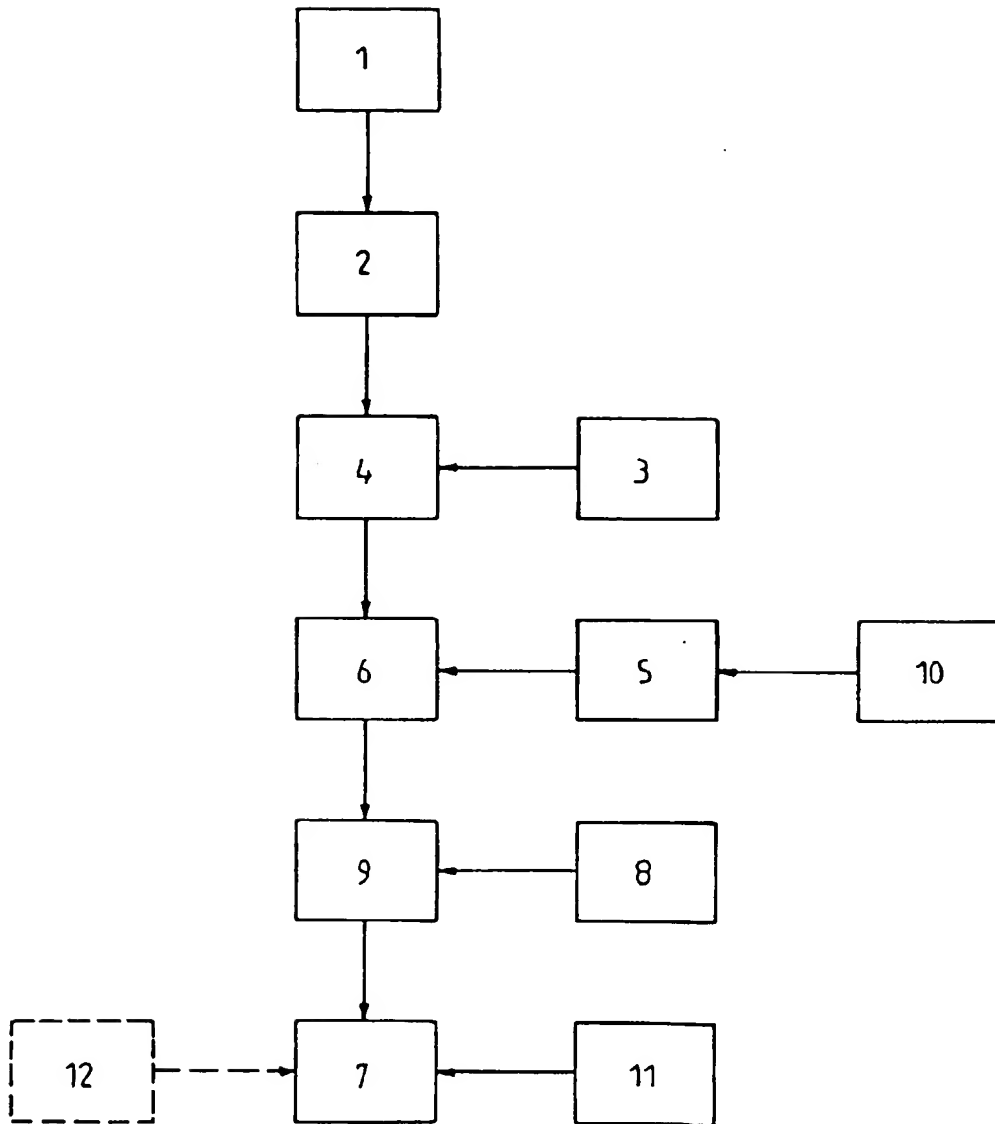


Fig. 3

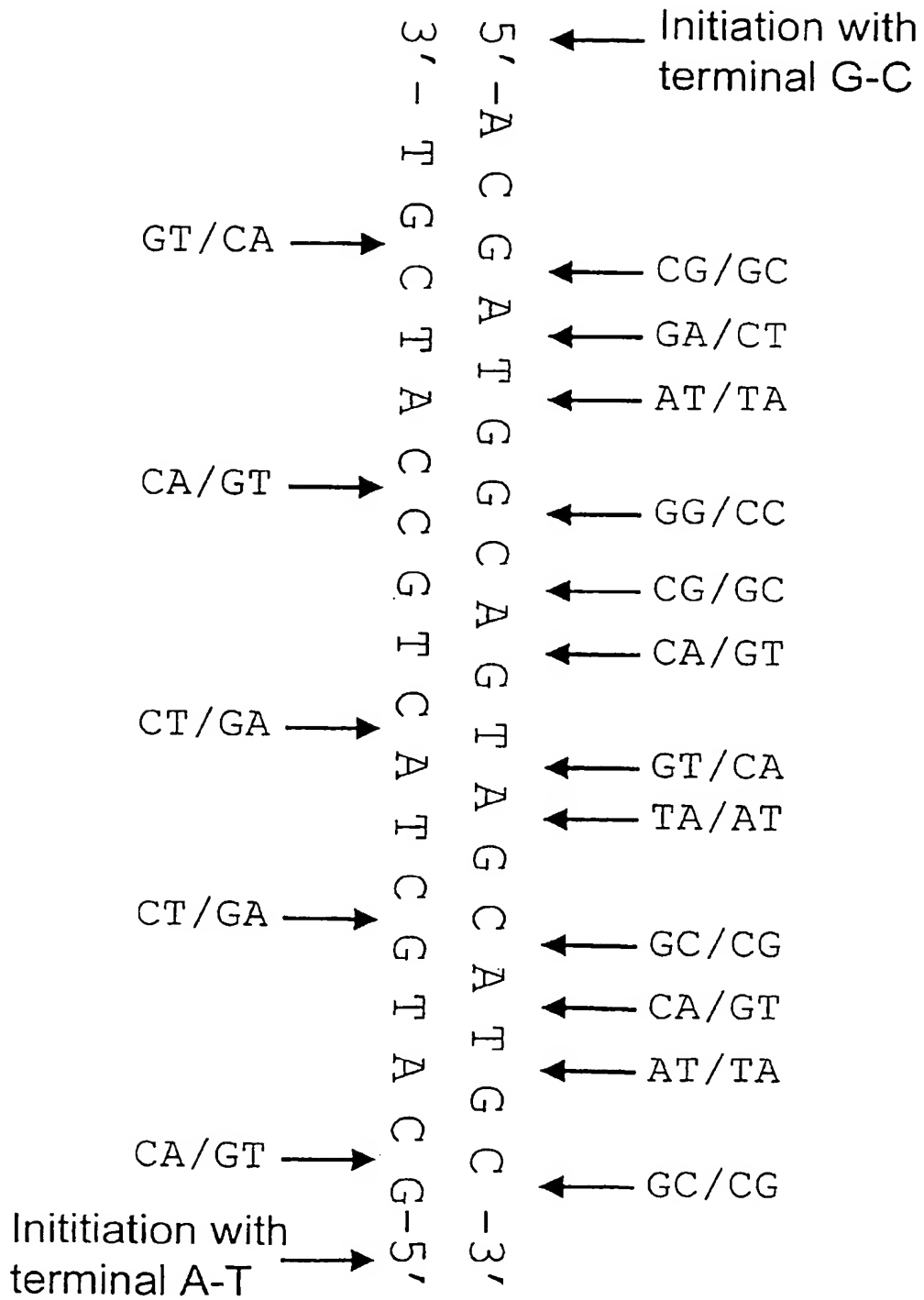


Fig. 4

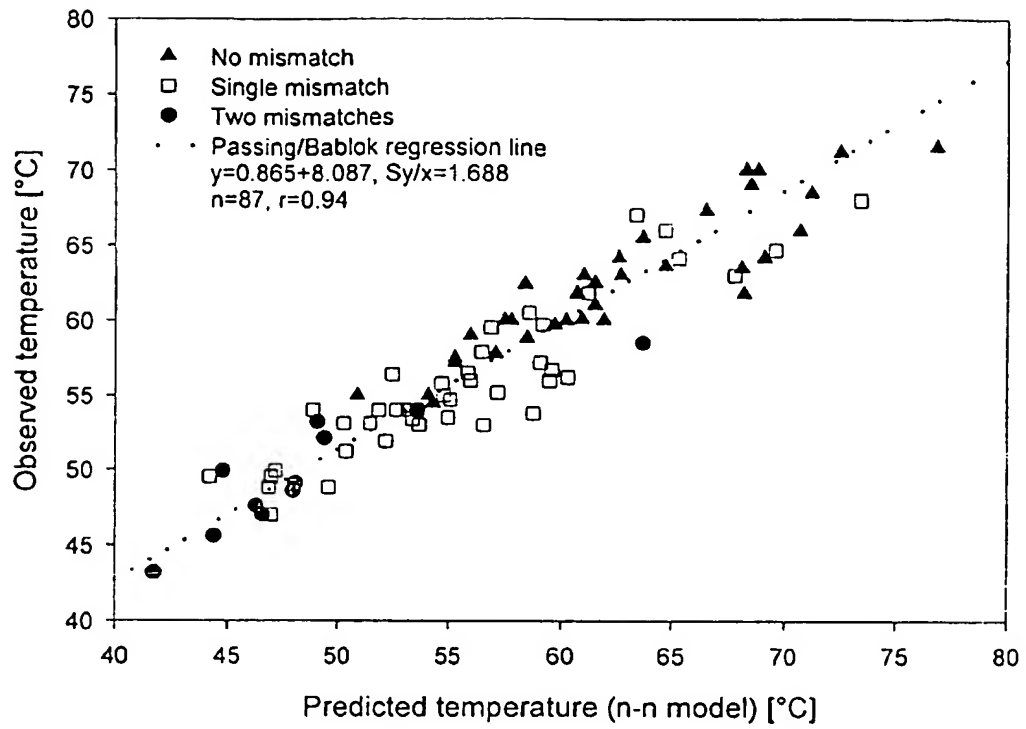


Fig. 5

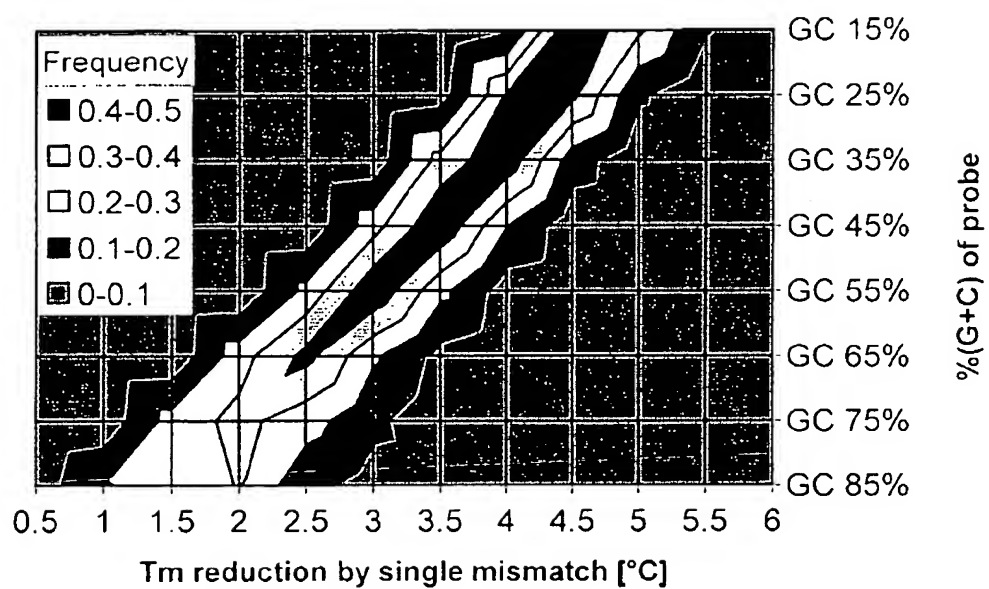


Fig. 6



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# EUROPEAN SEARCH REPORT

Application Number  
EP 99 12 3393

DOCUMENTS CONSIDERED TO BE RELEVANT.			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	US 5 856 103 A (CLARK CHRIS L ET AL) 5 January 1999 (1999-01-05)	1-3,7-10	G06F19/00
Y	* claims 1,6-8,11-15 * * column 4, paragraph 1 * * column 5, line 11 - line 30 * ---	4-6	
Y	US 5 556 749 A (MITSUHASHI MASATO ET AL) 17 September 1996 (1996-09-17) * abstract; claims 1-97 * ---	1-3,7-10	
Y	SANTALUCIA J ET AL: "IMPROVED NEAREST-NEIGHBOR PARAMETERS FOR PREDICTING DNA DUPLEX STABILITY" BIOCHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. EASTON, PA, vol. 35, 1 January 1996 (1996-01-01), pages 3555-3562, XP002062487 ISSN: 0006-2960 * abstract * * page 3561, left-hand column, paragraph 4 - right-hand column, paragraph 1 * ---	1-10	
A	GIESEN U ET AL: "A formula for thermal stability (T <sub>m</sub> ) prediction of PNA/DNA duplexes" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 26, no. 21, 1 January 1998 (1998-01-01), pages 5004-5006, XP002096183 ISSN: 0305-1048 * the whole document * --- -/--	1-10	<div>TECHNICAL FIELDS SEARCHED (Int.Cl.7)</div> <div>G06F</div>
The present search report has been drawn up for all claims			
Place of search <b>THE HAGUE</b>		Date of completion of the search <b>23 May 2000</b>	Examiner <b>Filloy García, E</b>
<div>CATEGORY OF CITED DOCUMENTS</div> <div> X : particularly relevant if taken alone  Y : particularly relevant if combined with another document of the same category  A : technological background  O : non-written disclosure  P : intermediate document  T : theory or principle underlying the invention  E : earlier patent document, but published on, or after the filing date  D : document cited in the application  L : document cited for other reasons  &amp; : member of the same patent family, corresponding document </div>			

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# EUROPEAN SEARCH REPORT

Application Number  
EP 99 12 3393

DOCUMENTS CONSIDERED TO BE RELEVANT			
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A	WO 98 18814 A (HALITSKY DAVID ; CUMULATIVE INQUIRY INC (US); FRESCO JACQUES R (US)) 7 May 1998 (1998-05-07) * page 2, line 25 - line 35 * * page 6, line 8 - line 37 *	1-10	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
The present search report has been drawn up for all claims			
Place of search <b>THE HAGUE</b>		Date of completion of the search <b>23 May 2000</b>	Examiner <b>Filloy Garcia, E</b>
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